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A Simple and Rapid Method for the Preparation of Adenosine Triphosphatase from Submitochondrial Particles

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An almost pure form of the bovine heart mitochondrial adenosine triphosphatase (ATPase) is released from the membrane by shaking submitochondrial particles with chloroform. Analyses on polyacrylamide gels and by electron microscopy, and also sensitivity to inhibitors, show that the chloroform-released enzyme is similar to other ATPase preparations from bovine heart mitochondria.

Appreciation of the probable role of the mitochondrial ATPase‡ in ATP synthesis associated with electron transport has led to the isolation and purification of the enzyme by different procedures from a variety of animal tissues and from *Saccharomyces cerevisiae*. There has been variation in the specific activity of the final products and in the reported subunit structure (Senior, 1973; Beechey, 1974; Munn, 1974; Kozlov & Mikelsaar, 1974), but consistent features of these preparations are the general complexity and length of the isolation procedures, and the large quantities of mitochondria used (Beechey & Cattell, 1973).

In this report we present an easy, rapid method for the extraction of the bovine heart mitochondrial ATPase and some properties of the isolated enzyme.

Experimental

Bovine heart mitochondria were prepared by the method of Sanadi & Fluharty (1963). Submitochondrial particles were prepared as described by Hansen & Smith (1964).

Protein was assayed by a modification of the method of Schachterle & Pollack (1973). To 75μl of the protein sample or blank was added 75μl of 6% (w/v) sodium deoxycholate and then 150μl of the alkaline copper reagent (Schachterle & Pollack, 1973). The sample was mixed and left for 10min at room temperature (17°C), then the solution was treated with 600μl of diluted Folin–Ciocalteu phenol reagent [BDH Chemicals Ltd., Poole, Dorset, U.K.; the reagent (1ml) was diluted to 22.5ml with water and used immediately], mixed well and incubated at 55°C for 5min. The colour was measured at 740nm. Bovine serum albumin was used to construct a standard curve.

ATPase activity at 30°C was assayed by measuring the rate of ADP production (Pullman *et al.*, 1960).

Polyacrylamide-gel electrophoresis was carried out with either 5% (w/v) gels (Knowles & Penefsky, 1972*a*), except that ATP and EDTA were omitted from the gels, or 10% (w/v) gels containing 0.1% sodium dodecyl sulphate (Weber *et al.*, 1972). Molecular weights were estimated on gels containing sodium dodecyl sulphate. Bovine serum albumin, trypsin, pepsin and cytochrome *c* were used to calibrate the gels. Protein bands were detected by staining with Coomassie Blue.

ATPase activity in the gels was detected by the method of Wachstein & Meisel (1957).

Results and Discussion

Release of the ATPase from submitochondrial particle membranes

Submitochondrial particles were suspended in a solution containing 0.25M-sucrose, 10mM-Tris sulphate and 1mM-EDTA, pH7.5–7.7, to give a final protein concentration of 5mg/ml. Analytical-grade chloroform (0.5vol.) was added and the two phases were mixed vigorously for 10–30s at room temperature. The emulsion was broken by centrifuging in a bench centrifuge at low speed. The aqueous layer thus obtained was then centrifuged at 10^5g for 30min at 20°C. The supernatant contained ATPase activity.

This procedure is amenable to large variations in scale. As a routine, small-scale preparations were

‡ Abbreviation: ATPase, adenosine triphosphatase (EC 3.6.1.3).

performed by using 2.5ml of submitochondrial particles containing 5mg of protein/ml. Successful extractions have been done by using up to 500mg of submitochondrial-particle protein. The yield of ATPase protein is 3–10% of the initial membrane protein used and the specific activity of the ATPase is 15–20μmol·min^{-1}·mg of protein^{-1} at 30°C. The apparent recovery of total ATPase activity is 50–60%. This yield is not precise because of the lack of information on the degree of association of the naturally occurring inhibitor protein (Pullman & Monroy, 1963) with the membrane-bound and the soluble ATPases. The ATPase solution can be concentrated by ultrafiltration by using Diaflo XM100A membrane filters. The ATPase protein may also be precipitated with $(NH_4)_2SO_4$ added to 60% saturation at 0°C. The resultant suspension can be stored at 0–4°C for several weeks without loss of enzymic activity.

The release of the ATPase molecule from the mitochondrial membrane by chloroform is surprisingly specific. Comparable experiments were performed with diethyl ether (Broughall *et al.*, 1973), di-isopropyl ether, carbon tetrachloride, ethyl acetate, butyl acetate, butan-1-ol and *n*-pentane; none of these solvents led to the dissociation of an active ATPase from the membrane.

Optimum conditions for release of the ATPase from the membrane

The release of ATPase is markedly sensitive to the pH of the submitochondrial-particle suspension over the range 6–9. Optimum dissociation from the membrane occurs at pH7.6. At the extremes of the pH range tested the amount of ATPase released was only 20% of that solubilized at pH7.6.

The composition of the medium in which the submitochondrial particles are suspended affects the amount and the specific activity of the solubilized ATPase (see Table 1). The presence of P_i and/or ADP lowers the specific activity of the ATPase; succinate had little effect in the presence of ADP and P_i. The highest specific activity and yield of solubilized ATPase were obtained in the presence of EDTA. The presence of ATP in addition to EDTA results in a low yield of ATPase with a high specific activity. In other experiments it was shown that the release of ATPase is inhibited by the presence of 2mM-Mg^{2+}. Thus chloroform treatment of submitochondrial particles suspended in a solution containing 0.25M-sucrose, 10mM-Tris sulphate and 1mM-EDTA, pH7.6, was adopted as a standard procedure.

There is little ATPase activity remaining in the membrane fraction after chloroform treatment. The chloroform treatment is either inactivating the membrane-bound ATPase activity or is very effective in dissociating the ATPase molecule from the membrane. We favour the latter explanation, because of the yields of protein released and the purity of the released ATPase.

Analysis of the ATPase preparation

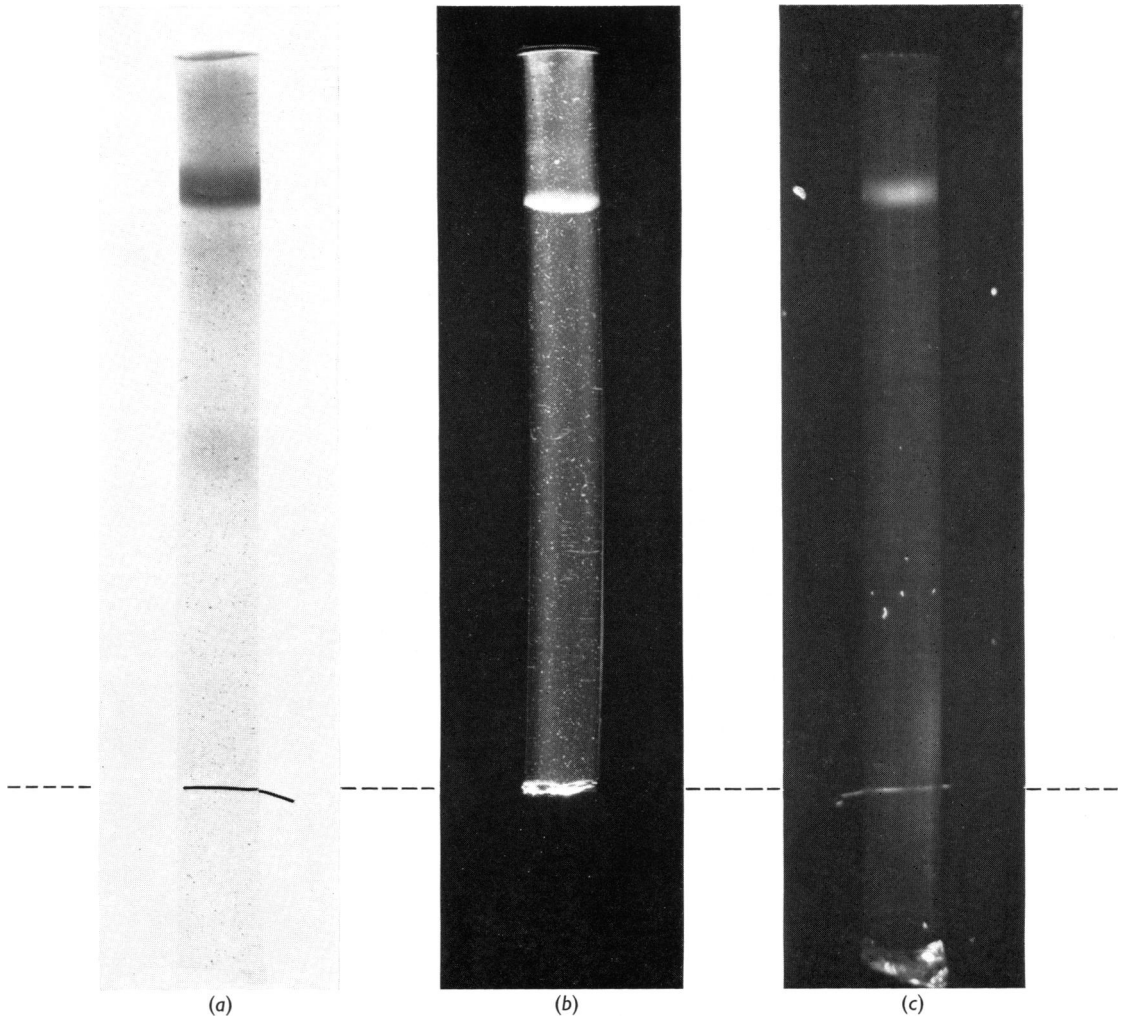
The aim of these experiments was to investigate the complexity of the membrane components released by treatment with chloroform.

Polyacrylamide-gel electrophoresis. The resolution of the released membrane components after electrophoresis on 5% (w/v) polyacrylamide gels was examined by three criteria: (1) staining with Coomassie Blue, (2) detection of ATPase activity and (3) the distribution of fluorescent material on the gel after pretreatment of the ATPase solution with aurovertin B (Osselton *et al.*, 1974; Mulheirn *et al.*, 1974). Aurovertin B is an inhibitor of the mitochondrial ATPase activity which binds specifically

Table 1. *Effects of different suspension media on the chloroform-induced release of the mitochondrial ATPase*

Submitochondrial particles (4.4mg of protein; specific activity 1.6μmol·min^{-1}·mg of protein^{-1}; total activity 7.0μmol·min^{-1}) were suspended in 0.25M-sucrose–10mM-Tris sulphate, pH7.7. Where indicated, ADP, ATP, P_i, EDTA and succinate were added to give a final concentration of 1mM. The final volume was 2.0ml; chloroform (1.0ml) was added. The tubes were vortexed for 10s at room temperature. The chloroform–water emulsion was broken by centrifugation in a bench centrifuge for 2min. The aqueous layer was centrifuged at 10^5g for 30min. The protein content and ATPase activity of the supernatant were assayed.

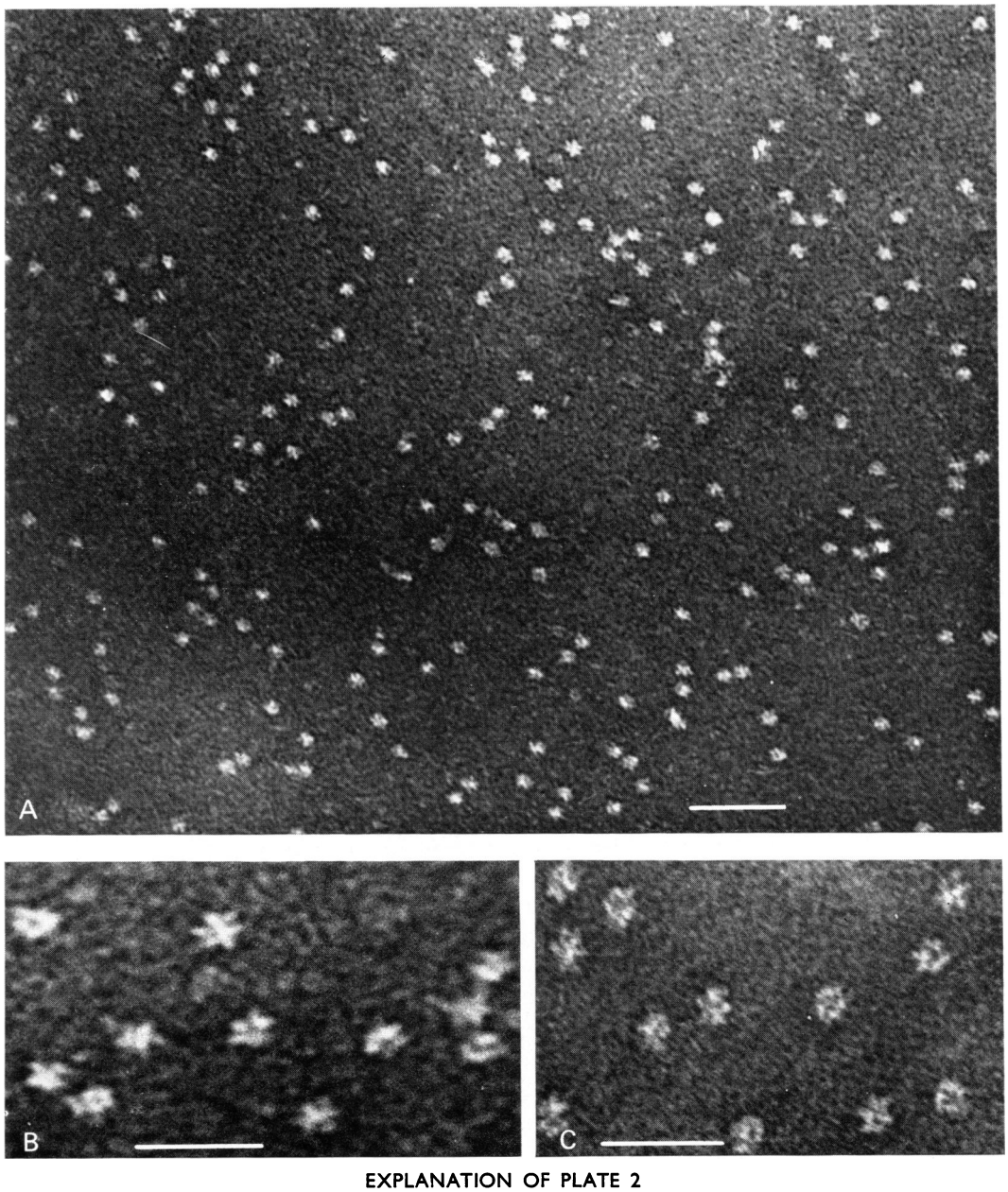
Additions to sucrose–Tris medium	Total protein released (mg)	Total ATPase activity (μmol·min^{-1})	ATPase activity (μmol·min^{-1}·mg of protein^{-1})
None	0.18	2.6	14.7
ADP	0.23	2.3	9.7
P_i	0.20	2.7	13.4
EDTA	0.19	3.7	20.1
ADP+P_i+EDTA	0.23	2.2	9.5
ADP+P_i+EDTA+succinate	0.18	2.2	12.1
ATP+EDTA	0.08	1.8	22.0



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of the chloroform-released ATPase

Chloroform-released protein (35μg) was electrophoresed on 5% gels. ----, Position of the Bromophenol Blue band. Gel (*a*), stained with Coomassie Blue. Gel (*b*), detection of ATPase activity. Gel (*c*), formation of a fluorescent complex with aurovertin B. The ATPase was treated with 1.8nmol of aurovertin B for 5min at 30°C before electrophoresis. The band was detected by irradiation at 365nm and viewed through a Wratten 2A filter (which is transparent to light above 405nm).



EXPLANATION OF PLATE 2

Electron micrographs of ATPase preparations negatively stained with 2% *sodium phosphotungstate, pH*7.4

Samples A and B are from a preparation obtained by the technique described in this paper. Sample C is from a preparation obtained by the technique of Senior & Brooks (1971), by P. V. Blair and E. A. Munn. The scale bar for A represents 50nm; those for B and C represent 25nm.

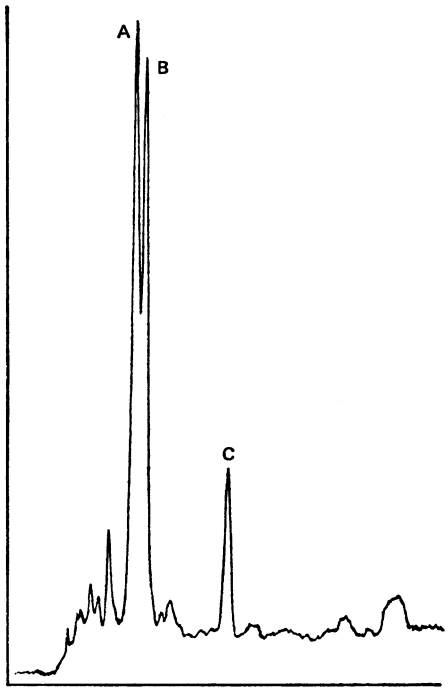


Fig. 1. *Subunit composition of the chloroform-released ATPase*

The ATPase was dissociated into subunits by treatment at 100°C with 1% mercaptoethanol and 1% sodium dodecyl sulphate, pH7.2, for 2min. Dissociated protein (10μg) was electrophoresed on 10% gels containing 0.1% sodium dodecyl sulphate. Protein bands were detected with Coomassie Blue and recorded with a Joyce-Loebl scanner. For details of bands A–C see the text.

to the enzyme molecule forming a fluorescent complex, in the same manner as aurovertin D (Lardy & Lin, 1969; B. Chance, H. A. Lardy & B. Schoener, unpublished work, quoted in Lardy & Ferguson, 1969).

Gel (*a*) (Plate 1) shows that most of the materials that stain with Coomassie Blue migrate as a single band. Gel (*b*) shows that ATPase activity is associated only with this major band. In gel (*c*) the aurovertin-binding material has the same electrophoretic mobility as the ATPase activity and the major Coomassie Blue-staining band. These results suggest that the major constituent of the chloroform-released components is the mitochondrial ATPase molecule.

The ATPase was denatured by heating for 2min at 100°C after the addition of mercaptoethanol and sodium dodecyl sulphate (Weber *et al.*, 1972). Fig. 1 shows the distribution of Coomassie Blue-staining material after electrophoresis of the denatured ATPase preparation on a 10% (w/v) polyacrylamide gel containing 0.1% sodium dodecyl sulphate. The trace accurately reflects the bands present on the gel. The distribution of protein and the approximate molecular weight of the three major bands, A, B and C, were 57000, 52000 and 35000 respectively, and are similar to those reported for bovine heart F_1 ATPase (Senior & Brooks, 1971; Knowles & Penefsky, 1972*b*) and rat liver mitochondrial ATPase (Catterall & Pedersen, 1971). Overloading of the gels shows the presence of small amounts of other proteins in the preparations.

Electron microscopy. Electron microscopy of ATPase preparations negatively stained with sodium phosphotungstate revealed uniform fields of particles (Plate 2, A). The particles are similar in size (maximum, overall dimension 11nm) and subunit structure (Plate 2, B) to those present in negatively stained preparations of bovine heart F_1 ATPase (Kagawa & Racker, 1966) (Plate 2, C) and the ATPase isolated from *Saccharomyces cerevisiae* mitochondria (Tzagaloff & Meagher, 1971).

Ultracentrifugation. The constituents of the chloroform-released mitochondrial membrane components (5mg of protein/ml of 25mM-Tris sulphate, 1mM-EDTA, pH7.7) were centrifuged in a Spinco model E analytical ultracentrifuge by using a double-sector cell. The macromolecular constituents sedimented as a single band, $s_{20,w} = 14.1$S. Penefsky & Warner (1965) reported a value of 12.9S for the F_1 ATPase dissolved in 0.1M-KCl and Warshaw *et al.* (1968) found $s_{20,w} = 13.05$S for the factor A-type ATPase, measured on a sucrose gradient.

This result, combined with those of the electron-microscopic and polyacrylamide-gel-electrophoretic analyses, shows that the material released by chloroform treatment of mitochondrial membranes is mainly the mitochondrial ATPase molecule.

Sensitivity of the ATPase preparation to inhibitors

The membrane-bound mitochondrial ATPase activity is inhibited by low concentrations of the oligomycins (Lardy *et al.*, 1958, 1965), venturicidin (Walter *et al.*, 1967), *NN'*-dicyclohexylcarbodi-imide (Beechey *et al.*, 1966) and triethyltin sulphate (Aldridge, 1958). Aurovertin D (Lardy *et al.*, 1964) and aurovertin B (Osselton *et al.*, 1974) are excellent inhibitors of mitochondrial ATP synthesis, but less effective inhibitors of the membrane-bound ATPase system. However, when in solution and separated from the membrane the mitochondrial ATPase loses its sensitivity to oligomycin and the trialkyltins (Kagawa & Racker, 1966). In addition, an active soluble ATPase can be prepared from *NN'*-dicyclohexylcarbodi-imide-treated submitochondrial particles (R. B. Beechey, A. M. Roberton & I. G. Knight,

Table 2. *Sensitivity of the chloroform-released mitochondrial ATPase to various inhibitors*

The ATPase activity was assayed as described in the Experimental section. Except for *NN'*-dicyclohexylcarbodi-imide, the inhibitors were added to the submitochondrial particles suspended in the ATPase assay medium in μl quantities of solutions in ethanol; equal volumes of ethanol were added to the controls. *NN'*-Dicyclohexyl-carbodi-imide (0.5nmol) in 5μl of ethanol was added to 0.1nmol of ATPase in 0.1ml of a solution containing 0.25M-sucrose, 10mM-Tris sulphate and 1mM-EDTA, pH7.6, and left at room temperature overnight. Ethanol was added to a control sample of ATPase. The ATPase activity of these samples was then assayed. A molecular weight of 3.5×10^5 for the ATPase was assumed, and the presence of contaminating proteins in the ATPase solutions was ignored in these calculations.

Inhibitor	Titre (mol of inhibitor/mol of ATPase)	Inhibition (%)
Oligomycin A+B	130	0
Venturicidin A	560	0
Triethyltin sulphate	3600	0
NN'-Dicyclohexylcarbodi-imide	6	87
Aurovertin B	18	45

unpublished work). However, the purified soluble ATPase still retains its sensitivity to the aurovertins (Kagawa & Racker, 1966; Roberton *et al.*, 1967). Penefsky (1967) showed that the incubation of purified soluble mitochondrial ATPase with *NN'*-dicyclohexylcarbodi-imide causes an inhibition of the enzyme activity. It must be emphasized that the ATPase is not the site of the inhibitory action of *NN'*-dicyclohexylcarbodi-imide when used to treat the membrane-bound ATPase (Cattell *et al.*, 1971).

Table 2 shows that the ATPase activity released from the mitochondrial membrane by treatment with chloroform is not affected by high concentrations of oligomycin, venturicidin A and triethyltin sulphate. It is, however, inhibited by *NN'*-dicyclo-hexylcarbodi-imide and aurovertin B. Thus the chloroform-released ATPase activity reacts to inhibitors in the same manner as the ATPase preparations released from the mitochondrial membrane by other procedures.

Whereas the chloroform-released ATPase has many properties in common with the mitochondrial ATPases prepared by other procedures, there are some differences. The specific activity of the chloroform-released ATPase is low when compared with the specific activity of approx. 100μmol$\cdot$min$^{-1}\cdot$mg of protein^{-1} shown by other preparations of bovine heart mitochondrial ATPase (Horstman & Racker, 1970; Senior & Brooks, 1970; Knowles & Penefsky, 1972*a*). The low specific activities could be due either to inactivation during the chloroform treatment or to the association of the mitochondrial ATPase-inhibitor protein (Pullman & Monroy, 1963) with the solvent-released ATPase. If the latter were true, the chloroform-released ATPase would be similar to factor A (Andreoli *et al.*, 1965), a mitochondrial ATPase preparation with a low specific activity. Another property shared by factor A and the solvent-released ATPase is the cold-stability of the enzymic activity, in contrast with the cold-lability shown by other bovine heart mitochondrial F_1 ATPase preparations (Pullman *et al.*, 1960). Combination with the inhibitor protein confers cold-stability on F_1 ATPase (Pullman & Monroy, 1963). The ATPase activity of factor A is increased by heating, but this is not so for the chloroform-released ATPase. However, we have noted that after precipitation with $(NH_4)_2SO_4$ the solvent-released ATPase becomes cold-labile after dissolving the precipitate.

The major merits of the procedure described here are speed, flexibility and simplicity. The ability to prepare ATPase from small quantities of sub-mitochondrial particles enables studies on ATPases from limited sources of biological material such as insect flight-muscle and brown-adipose-tissue mitochondria. The solubilization of the mitochondrial ATPase by using acidic phospholipid micelles (Toson *et al.*, 1972) can also be used to prepare small amounts of ATPase. However, although it is effective, the procedure is more involved than that described here, and certainly more expensive.

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